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(54) Title: METHODS AND USES FOR APOPTIN

(57) Abstract

　　This invention describes that apoptin fails to induce apoptosis in human normal cells. Besides, the invention describes that when normal cells are transformed, they become susceptible to the apoptin-induced apoptosis. The invention describes that apoptin induces in various human tumor cells a p53-distinct type of apoptosis, and cannot be blocked by a variety of apoptosis-inhibiting agents. The invention comprises an anti-tumor agent, which specifically kill tumor and not normal cells. It further provides the induction of cell death by means of gene therapy. Apoptin can induce apoptosis in non-human animal tumor cells. The invention also provides that in normal cells apoptin was found predominantly in the cytoplasm, while in tumor cells it was located in the nucleus. Besides, the invention comprises a diagnostic test for the determination of cell-transforming activity.

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Methods and uses for apoptin.

BRIEF DESCRIPTION OF THE INVENTION.

The present invention relates to methods and uses for
apoptin or derivatives or functional fragments thereof,
whereby apoptin stands for the viral protein 3 (VP3) of
the chicken anaemia virus. It furthermore provides novel
5   derivatives of said apoptin. Apoptin itself and its
apoptosis inducing activity have been disclosed before
(see below). However, in those earlier publications
apoptin was no different from any other apoptosis inducing
10   agent. We have now found that apoptin is indeed very
different from other apoptosis inducing agents and may
therefore be applied in different methods and for
different uses.Whereas conventional apoptosis inducing
agents induce apoptosis in any cell in which they are
15   present we have now found that apoptin induces apoptosis
only in transformed cells or tumor cells.

Thus the invention in one embodiment provides anti-
tumor therapies. The application of apoptin as anti-tumor
therapy will cause little toxicity for apoptin induces
20   cell death to a high extent in tumor cells and much
reduced if at all in normal non-transformed, non-malignant
cells.

The invention also discloses that apoptin cannot be
inhibited by several anti-apoptosis inhibitors. In
25   particular, apoptin acts distinct from the p53-apoptotic
pathway, which is known to be a necessary element in
mediating apoptosis, which is triggered by a variety of
(chemo)-therapeutic agents.

The invention further discloses the finding that
30   apoptin can induce apoptosis in various types of mammalian
tumor cells.

The invention further relates to differences in
localization of apoptin in cells susceptible for apoptin-
induced apoptosis, viz. human transformed and malignant

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cells, versus cells insensitive to apoptin-induced
apoptosis, viz. human normal cells. The differential
location is used as diagnostic assay to analyse whether a
cell is normal or has become transformed and/or malignant.

BACKGROUND OF THE INVENTION.

Jeurissen et al. (1992b) observed a number of
phenomena in the thymi of chicken anemia virus (CAV)-
inoculated specific-pathogen-free chickens that were
absent in thymi of control chicken. Ten days after
infection, the entire cortex contained cells whose
chromatin had condensed in areas adjacent to the nuclear
membrane. More or less spherical electron-dense bodies
were seen sporadically in the cytoplasm of epithelial
cells. At day 13 after infection, the cortex was severely
depleted of thymocytes, whereas epithelial cells, many of
them containing electron-dense bodies and other non-
lymphoid cells were still present. DNA isolated from
thymuses of chickens infected with various field isolates
of CAV displayed the typical laddering of oligonucleosomal
breakdown in an electropherogram. These observed
morphological cellular and biochemical changes were also
observed in CAV-infected cultured avian lymphoblastoid
cells.

The above phenomena are characteristic of the
physiological process of programmed cell death or
apoptosis. Apoptosis is characterized by shrinkage of
cells, segmentation of the nucleus, condensation and
cleavage of DNA into domain-sized fragments, in most cells
followed by internucleosomal degradation. Finally, the
apoptotic cells fragment into membrane-enclosed bodies,
which are rapidly phagocytosed by neighboring cells.
Therefore, apoptosis causes much less destruction of
tissue than necrosis, the non-physiological type of cell
death (Wyllie et al., 1980, Arends and Wyllie, 1991).

Apoptosis is a cascade of events. In general, the
apoptotic process can be devided in several stages.

Stage 1. Triggering of apoptosis.
Many different external and internal agents can trigger
the apoptotic process.

Stage 2. Factors which can mediate the apoptotic
trigger.
A main role at this stage is played by e.g. the tumor-
suppressor protein p53.

Stage 3. Enhancing of the apoptotic signal by
factors as Bax.

Stage 4. Activation of the ICE serine-protease
family members.
As soon as these specific proteases are activated, the
point of no return has been passed. Known apoptosis
inhibitors as growth factors (inhibits at stage 2), Bcl-2
(stage 3) or crmA (stage 4) are known to inhibit
apoptosis at different stages during the decission stage
of the apoptotic process

Stage 5. Execution of the apoptotic signal. For
instance, DNA becomes condensed and
fragmented.(White, 1996).

       Early after infection of cultured chicken mononuclear
cells, the CAV-encoded protein apoptin (also called VP3)
co-localizes with cellular chromatin. Later after
infection, apoptin forms aggregates, the cells become
apoptotic, i.e. the cellular DNA is fragmented and, as a
result, becomes condensed (Noteborn et al., 1994). It was
shown by immunogold electron microscopy that apoptin is
present in apoptotic structures. In vitro, expression of
apoptin in chicken transformed lymphoblastoid T cell and
myeloid cell induced apoptosis in these cells. These data
indicate that apoptin can trigger the apoptotic pathway in

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CAV-infected cells (Noteborn et al. 1994, Noteborn and
Koch, 1995).

Apoptin is a small protein, only 121 amino acids
long, which is rather basic and proline-rich (Noteborn et
5   al., 1991). Apoptin is located strictly within the
cellular chromatin structures. Truncation of the C-
terminal basic stretch of apoptin results in a reduced
nuclear location and a significantly reduced apoptotic
activity (Noteborn et al., 1994). The small size and
10   rather basic character of apoptin may allow it to interact
with histone and/or non-histone proteins within the
chromatin structure.

Apoptosis is an active and programmed physiological
process for eliminating superfluous, altered or malignant
15   cells (Earnshaw, 1995). The apoptotic process can be
initiated by a variety of regulatory stimuli (Wyllie, 1995
and White, 1996). Changes in the cell survival rate play
an important role in human pathogenesis, e.g. in cancer
development, which is caused by enhanced cell
20   proliferation but also by decreased cell death (Kerr et
al., 1994). A variety of chemotherapeutic compounds and
radiation have been demonstrated to induce apoptosis in
tumor cells, in many instances via wild-type p53
(Thompson, 1995, Bellamy et al., 1995, Steller, 1995,
25   Kaufman, 1989, McDonell et al., 1995, Lowe et al. and
Fisher, 1994).

Many tumors, however, acquire a mutation in p53
during their development, often correlating with poor
response to cancer therapy (Hooper, 1994). For several
30   (leukemic) tumors, a high expression level of the proto-
oncogene Bcl-2 is associated with a strong resistance to
various apoptosis-inducing chemotherapeutic agents
(Hockenberry, 1994, Kerr et al., 1994, Sachs and Lotem,
1993).
35   Apoptin can induce apoptosis human malignant cell
lines (Noteborn and Koch, 1994). We have established that
apoptin-induced apoptosis occurs in the absence of

functional p53 (Zhuang et al., 1995a), and cannot be
blocked by Bcl-2 and BCR-ABL (Zhuang et al., 1995).
Therefore, apoptin is usefulfor the destruction of tumor
cells, which have become resistant to (chemo)therapeutic
induction of apoptosis, due to the lack of functional p53
and (over)-expression of Bcl-2 and BCR-ABL.

DETAILED DESCRIPTION OF THE INVENTION.

The invention discloses the apoptotic activity of
apoptin in (e.g.human) malignant and transformed cells
versus normal cells. Apoptin fails to induce apoptosis in
primary T cells, endothelial and smooth muscle cells. When
normal cells are transformed they become susceptible by
apoptin. In normal cells apoptin was found predominantly
in the cytoplasm, while in tumor cell it was located in
the nucleus.

Apoptin can be used for depletion of specifically
transformed/malignant cells. Therefore, apoptin is a very
potent anti-tumor agent. Expression of apoptin can be
used for the induction of apoptosis in specifically
(human) tumor cells. Apoptin does not or at least not
significantly induce apoptosis in normal cells, indicating
that the toxicity of apoptin-treatment will be low.

The invention discloses that apoptin is able to
induce apoptosis in transformed, non-immortalized cells,
which implies that the apoptotic activity of apoptin
becomes already available during early transformation
events in cells. Therefore, apoptin can be included in
other (e.g. gene- or chemo-) therapies to prevent treated
cells for undergoing transformation and even malignancy.

Apoptin can be (transiently) expressed in tumors by
means of DNA transfection. Expression of apoptin in
(tumor) cells may also take place by infecting cells with
(retro)viral vectors that contain a coding sequence for
apoptin.

Administration to cells of non-viral components (e.g.
liposomes or transferrin-derived vectors) containing

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apoptin proteins and/or coding sequence for apoptin is a
further possibility for the expression/presence of apoptin
and induction of apoptosis in tumor cells.

Furthermore, we have provided evidence that apoptin
5   is distinct from the p53-induced apoptotic pathway.
Besides the fact that apoptin does not need functional
p53, apoptin cannot be blocked by inhibitors of the p53-
apoptotic pathway, as the Bcl-2(-like) proteins, and the
cow-pox protein crmA, which blocks the ICE-like protease
10   activities. These inhibitors interfere within different
stages of the p53-induced apoptotic cascade. Apoptin,
might induce apoptosis completely independent from the
apoptic pathway
blocked by these apoptotic inhibitors, or act downstream
15   from them.

These data imply that apoptin is a very potent
inducer of apoptosis in tumor cells, which can overcome
(all analysed) blocks of anti-apoptotic activity in
transformed
20   and malignant cells. Therefore, apoptin is a potent anti-
tumor agent for a braod variety of tumor(s) (cells).

The differential localization of apoptin in normal
versus transformed/malignant cells can be used as
diagnostic test to distinguish whether a cell has become
25   transformed/malignant.

Besides, the induction of apoptosis in human tumor
cells, we have also provided evidence that apoptin can
induce apoptosis in other mammalian tumor cells. The
invention can be used for treatment of cancer in various
30   mammalian sytems.

Thus the invention provides a vehicle delivering a
tumoricidal substance or a gene encoding a tumoricidal
substance mainly but not exclusively with a tumor cell,
characterized in that the tumoricidal substance is apoptin
35   or a functional equivalent thereof. In the prior art many
vehicles for delivering cytotoxic agents or precursors
therefor have been disclosed. A major problem in

delivering cytotoxic agents is that they are harmful to
all cells and not just the tumor cells (which will be
interchangeably called transformed or malignant or tumor
cells herein). Therefore many different ways of targetting
5   the cytotoxic substance to the tumor cells have been
investigated. Although many targetting moieties are
nowadays known, all of them suffer from the drawback of
not being completely specific for the tumor target.
Therefore the use of this cytotxic substances has sofar
10   not met with great success. At the time of its discovery
apoptin was thought to suffer from the same drawback in
that it would need a highly specific targetting moiety. We
have now found that this is not necessary for apoptin.
Apoptin only has a significant effect in tumor cells and
15   not in normal cells. Thus, even if the targetting moiety
or any other means of delivery of apoptin (or a functional
equivalent) is not very specific, this will result in
hardly any toxicity to normal tissue. Thus the invention
provides a conjugate for targeted tumor therapy comprising
20   a targeting moiety having binding affinity for a molecule
associated mainly, but not exclusively with the surface of
a tumor cell and apoptin or a functional equivalent
thereof. A functional equivalent of apoptin is any
fragment or derivative having the same kind of activity,
25   possibly in different amounts.
        A targetting moity is well defined in the art as a
molecule with a specific binding activity for a target
molecule. It should preferably be capable of
internalization. The target molecule may be an antigen or
30   an epitope, in which case the targetting moity is an
antibody or a fragment or a derivative thereof. The target
molecule may be a receptor in which case the targetting
moiety is a ligand for said receptor. These are just
examples of suitable combinations. The bond between the
35   targetting moiety and the apoptin has only one requirement
in that it should allow the functions of both partners to
operate. Thus, it may be a chemical (labile) bond, it may

be a fusion protein. The conjugate may even be a liposome
covered with targetting moieties and filled with apoptin
(or a gene encoding it) etc. The invention also comprises
a vehicle delivering a gene encoding apoptin to a tumor

5   cell, using gene therapy. Gene therapy has many well known
methods to deliver genes to cells using viruses such as
adenovirus or retrovirus. The person skilled in the art
will know how to select the right vehicle. Because apoptin
only functions to induce apoptosis in transformed cells,

10   it or its gene can be used as a safety measure in other
gene therapy regimens than tumor therapy, such as those
rectifying deficiencies from inheritable diseases. It is
then provided in a gene delivery vehicle together with the
gene of interest and if the cell in whhich the gene of

15   interest is inserted becomes malignant then it will be
susceptible to the action of apoptin. Thus the invention
provides a vehicle for delivering a nucleic acid of
interest to a target cell, said vehicle further comprising
a gene encoding apoptin or a functional equivalent

20   thereof.
       Since this is the first really feasible medical use
of apoptin such a use is also part of the present
invention. Thus there is provided apoptin or a functional
equivalent thereof for use in a method of eliminating

25   cells of a target cell population whereby the method is
mainly, but not entirely specific for the cells of the
target population, whereby apoptin or its functional
equivalent is the ctotoxic agent, as well as apoptin or a
functional equivalent thereof for use in a method of

30   eliminating cells of a target cell population whereby the
cells of said population are not sensitive to other
apoptosis inducing agents.
       The latter is possible because apoptin cannot be
inhibited by the mechanims inhibiting the other apoptosis

35   inducing substances. If apoptin is to be used to eliminate
cells that are not transformed then this can be achieved
by providing apoptin or a functional equivalent thereof

provided with a nuclear localization signal. Since apoptin
needs to be in the nucleus this will result in apoptosis.
Without said signal the apoptin would remain outside the
nucleus and have no significant effect (as disclosed

5   herein).

       Thus said apoptin with a nucleus localization signal
could be used in a method for elimination of cells of a
population of target cells. Nuclear localization signals
(NLS) are well known in the art. One could also provide a

10   fusion gene encoding an NLS and an apoptin.

       Because apoptin has a different localization in
normal cells as compared to in transformed cells, it is
possible to use apoptin as a diagnostic tool
distinguishing between those two kinds of cells. Thus the

15   invention also provides a method for distinguishing
between transformed and/or malignant and/or tumor cells
and normal cells comprising the steps of providing said
cells with the viral protein 3 (VP3; apoptin) and
detecting the localization of said viral protein in said

20   cells. The localized apoptin can be detected with e.g.
antibodies.

       The invention will be explained in more detail on the
bassis of the following experimental part. This is only
for the purpose of illustration and should not be

25   interpreted a a limitation of the scope of protection.

       EXPERIMENTAL

       Isolation of human primary cells.

30   Human primary T cells were isolated from 6 normal
blood donors by Ficol centrifugation and grown in RPMI-
medium containing 6% human serum and 0.8 ug per ml
phytohemagglutinin. After 3 days the medium was refreshed
and 300 units per ml Interleukin-2 was added. Human

35   primary smooth-muscle cells (SMC) and vascular endothelial
cells (HUVEC) were isolated from umbilical cords. SMC were

grown in CM199 medium supplemented with ECGF and heparine
and HUVEC in DMEM medium containing 10% fetal calf serum.

DNA plasmids.

All CAV DNA sequences are originally derived from the
plasmid DNA pIc-20H/CAV-EcoRI (Noteborn and De Boer,
1990). All cloning steps with plasmid DNAs were in
principle carried out according the methods by Maniatis et
al. (1982).

The expression plasmid pCMV-fs, formerly called pCMV-
VP3, contains CAV DNA sequences encoding apoptin
exclusively (nt 427-868), plasmid pCMV-tr encodes a
truncated apoptin which lacks the C-terminal 11 amino
acids (Zhuang et al., 1995a), and pCMV-des encodes desmin,
a structural protein found in muscle cells that does not
induce apoptosis (Menke et al., unpublished data). The
expression plasmids pCMV-BAG-1 (Noteborn, M.H.M.,
unpublished data), pCMV-BcL-2 (Zhuang et al., 1995b) and
pCMV-crmA (Noteborn, M.H.M., unpublished data) expresses
the anti-apoptosis proteins BAG-1, Bcl-2 and the cowpox
crmA protein inhibiting ICE-like proteases, respectively.
The expression plasmids pCMV-E1B21K expresses the
adenovirus E1B 21kDa protein and pCMV-p53, the tumor
supressor protein p53 (Zhuang et al., 1995).

All transiently expressed genes are under the
regulation of the cytomegalovirus promoter.

Human normal, transformed and malignant keratinocytes and fibroblasts.

Human keratinocytes were isolated from foreskin and
grown in the presence of a layer of mouse 3T3 fibroblasts
lethally irradiated with 137Cs. Primary cultures of human
epidermal keratinocytes (FSK-1) were initiated in complete
medium as described (Rheinwald and Green, 1975) with minor
modifications.

Human normal foreskin diploid VH10 fibroblasts
(Klein, 1990) were grown in DMEM supplemented with 10%
fetal-calf serum.

SV40-transformed tumorigenic NW18 fibroblasts
5   (Weissman and Stanbridge, 1983) were grown in MEM medium
supplemented with 8% fetal calf serum.

Tumorigenic keratinocytes, SCC-15 (Rheinwald and
Beckett, 1981), derived from squamous-cell carcinoma, were
cultured in DMEM/F12 (3:1) medium containing 5% fetal-calf
10 serum, 0.4 ug per ml hydrocortisone and 1 uM
isoproterenol.

The SV40-transformed fibroblasts, pre-crisis (Pre)
and post-crisis (Post), as described by B. Klein et al.
(1990) were grown in MEM medium with 8% fetal-calf serum.
15 The spontaneously transformed keratinocyte strain HaCaT
(Boukamp et al., (1988) was a gift from Prof. Dr. Fusenig,
DKFZ, Heidelberg, Germany. HaCaT cells were grown in DMEM
medium supplemented with 10% fetal-calf serum. The SV40-
transformed keratinocyte strain SVK14 (Taylor-
20 Papadimitriou et al., 1982) cells were cultured in the
same medium as SCC-15 cells.

DNA transfections.

Plasmid DNA was purified by centrifugation in a CsCl
25 gradient and column chromatography in Sephacryl S500
(Pharmacia).

Phytohemagglutinin-stimulated primary human T cells
were transfected in the presence of DEAE-dextran, as
described (Noteborn et al. 1994). Mouse Crip cells and
30 human Hep3B, VH10, Pre- and Post-, and NW18 cells, HUVECs
and SMCs were transfected with plasmid DNA by calcium-
phosphate precipitation as described (Graham and Van der
Eb, 1973). FSK-1, HaCaT, SVK14 and SSC-15 cells were
transfected with DOTAP [D.Fischer, unpublished results]

<u>Immunofluorescence</u>

T cells were grown in suspension and fixed on glass
microscope slides. All other cells were grown on coated
glass microscope slides. The cells were fixed with 80%
5   acetone for 10 min at room temperature, and used for
indirect immunofluorescence as described (Noteborn et al.,
1994). To demonstrate the presence and/or cellular
location of (truncated) apoptin in transfected cells mouse
monoclonal antibodies (MAb) CVI-CAV-85.1 (85.1) (Noteborn
10   et al., 1991) and CVI-CAV-111.3 (111.3; Koch, unpublished
data) were used and for human desmin the mouse MAb 33
(Monosan, Uden, The Netherlands) was used. Fluorescein-
isothiocyanate-labeled goat anti-mouse antibodies (Jackson
Immunoresearch Laboratories Inc., West Grove PA) were
15   used as second antibodies. Nuclear DNA was stained with
2,4-diamidino-2-phenylindole (DAPI) or propidium iodide
(PI).

<u>Stable DNA tranfection of VH10 cells.</u>
20   Human normal diploid VH10 fibroblasts were stably
transfected with pCMV-fs, expressing full-sized apoptin or
pCMV-neo-Bam, the empty plasmid without the CAV sequences
encoding apoptin. Stable clones were selected with G418
medium, grown on glass microscope slides and fixed with
25   80% acetone. Expression of apoptin was analysed by
indirect immunofluorescence using MAb 85.1.

RESULTS AND DISCUSSION

30   <u>Expression of apoptin in mouse tumor cells in vitro.</u>
We have examined whether apoptin can induce apoptosis
also in mammalian tumor cells of non-human origin.
Therefore, cells of the mouse tumor cell line Crip (Danos,
1988) werxe transfected with pCMV-fs DNA encoding apoptin.
35   Three days after transfection the cells were fixed. By
meansof immunofluorescence and PI staining the cells were
examined for expression of apoptin, and whether they

became apoptotic. Already, at three days after
transfection 54% of the mouse tumor cells, which contain
apoptin, have become apoptotic.

These results indicate that apoptin can induce
5   apoptosis in different mammalian tumor cell lines.

In Hep 3B cells apoptin-induced apoptotic pathway is
distinct from the p53-induced apoptotic pathway.

Recently, we have shown that apoptin induces
10   apoptosis in osteosarcoma cells, without the presence of
wild-type p53 (Zhuang, et al., 1995, Noteborn and Koch,
1994). Chiou et al. (1994) and Debbas and White (1993)
have provided evidence that Bcl-2 and adenovirus 5 (Ad 5)
E1B 21K protein can block p53-induced/mediated apoptosis
15   by acting downstream from p53.

We examined whether Ad 5 E1B 21K protein and Bcl-2
can inhibit the p53-independent pathway of apoptosis
induced by apoptin in comparison to p53-dependent
apoptosis. To that end, we studied the effect of co-
20   expression of these proteins with apoptin or p53 in the
human hepatoma Hep3B cell line.

Hep3B cells were co-transfected with pCMV-fs,
encoding apoptin and pCMV-neo-Bam DNA (negative control),
pCMV-E1B21 DNA, encoding the Ad5 E1B 21K protein or pCMV-
25   Bcl2 (Zhuang et al., 1995b), encoding human Bcl-2. The
number of apoptin-expressing cells was screened by
indirect immunofluorescence and by DAPI-staining, which is
weak and/or irregular when the cells have become
apoptotic.
30   Surprisingly, at several timepoints after
transfection the percentage of apoptin-expressing Hep3B
cells, which became apoptotic was similar to the Hep3B
cells containing both apoptin and E1B-21K or both apoptin
and Bcl-2. For the sake of brevity, only the data obtained
35   six days after transfection are given in Figure 1.

To illustrate that E1B-21K or Bcl-2 indeed have an
anti-apoptotic effect in Hep3B cells, we examined whether

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these two proteins could inhibit apoptosis caused by over-
expression of p53 in Hep3B cells. Compared to co-
transfection of Hep3B cells with pCMV-p53 DNA encoding
wild-type p53 (Baker et al., 1990) and pCMV-neo-Bam, co-
5   transfection with plasmids pCMV-p53 and pCMV-E1B21, or
pCMV-Bcl2 resulted in a significant reduction of apoptosis
induced by p53, as analysed by immunofluorescence and PI-
staining (Figure 2).

        Thus, absence of effect of E1B 21K and Bcl-2 proteins
10   on apoptin-induced apoptosis can not be explained by non-
functionality of the expressed proteins in Hep3B cells for
they can inhibit p53-induced apoptotic pathway. The fact
that E1B 21K and Bcl2 still could negatively influence the
p53-regulated apoptotic pathway in Hep3B cells, although
15   the apoptin-induced apoptosis could not be inhibited
indicates that the p53-dependent and apoptin-inducable
p53-independent apoptotic pathway are distinct routes at
least within Hep3B cells.

        The fact that in a large number of tumors apoptosis
20   cannot be induced in a variety of chemotherapeutic agents
seems to be related to disrupted functions of p53 (Lowe et
al., 1993). Therefore, induction of a p53-independent
apoptotic pathway is a useful approach as alternative for
tumor therapy.
25

Characterization of the apoptin-induced apoptotic pathway
in Saos-2 cells.

        The apoptin-induced apoptotic pathway seems to be
distinct from that induced by p53 (see above). Bcl-2,
30   known to be involved in tumorformation via inhibition of
the apoptotic pathway, could not block apoptin-induced
apoptosis, but could block the p53-pathway. Recently, it
has been reported by others that Bcl-2 and Bag-1 (Takayama
et al., 1995) together might be required for the
35   inhibition of so-called Bcl-2-independent apoptosis, as
seems to be the case for apoptin-induced apoptosis.

To examine whether Bag-1 could negatively influence the apoptin-induced apoptosis on its one or together with Bcl-2, co-transfections of Saos-2 cells pCMV-fs and pCMV-Bcl-2, or pCMV-Bag-1, or pCMV-Bcl-2 and pCMV-Bag-1 were

5   carried out. In parallel, a controls, similar co-transfection experiments were carried out with p53. Bag-1 or a combination of Bag-1 and Bcl-2 did not inhibit apoptin-induced apoptosis, whereas p53-induced apoptosis was significantly inhibited by Bag-1 or Bag-1 and Bcl-2

10   (Figure 3). We conclude that the apoptin-induced apoptotic pathway is independent from Bcl-2-like proteins or acts downstream from them.

By double-immunofluorescence and video-intensified fluorescence microscopy was analysed whether apoptin could

15   regulate bax-expression. Bax is a cellular protein, which can induce apoptosis and associates to Bcl-2 proteins and other, yet unknown, cellular proteins. p53 upregulates the expression of Bax, which results in induction of apoptosis (Oltvai et al. 1993). We have found evidence that apoptin

20   did not upregulate the Bax protein level, whereas in a similar experiment was shown that p53 could do so. This is another argument that apoptin seems not to act via the p53-apoptotic pathway.

Interleuking-1-beta-converting-enzyme (ICE)-like

25   proteins are known to be the last or one of the last steps in the decision cascade of the apoptotic process (Kumar, 1995). To examine whether ICE-like proteins might play a role in apoptin-induced apoptosis, the ICE-inhibitor crmA was co-expressed with apoptin. We have obtained evidence

30   that inhibition of the ICE-like proteins due to expression of the cowpox protein crmA does not result in inhibition of apoptin-induced apoptosis. However, in a parallel experiment it was shown that crmA expression could reduce p53-induced apoptosis (Figure 4).

35   Therefore, we conclude that apoptin is very close to or even beyond the point of no-return within the apoptotic decision cascade. Furthermore, apoptin-induced apoptosis

do not or only use a very minor part of the p53-induced
apoptotic pathway.

Expression of apoptin in normal human cells.

5   Three types of human primary cells, i.e. vascular
endothelial cells (HUVEC), smooth muscle cells (HSMC) and
T cells, were transiently transfected with a
plasmidencoding full-sized apoptin (pCMV-fs). Cells
expressing apoptin were screened via indirect
10   immunofluorescence with MAb 85.1[8] or 111.3. Induction of
apoptosis in apoptin-positive cells, was analysed with
the help of DAPI or PI, which stain intact nuclei
regularly, but apoptotic ones irregularly and/or weakly
(Telford, 1992). Five days after transfection, only around
15   20% of the apoptin-expressing primary cells stained
abnormally with DAPI or PI (data not shown). Menke et al.
(unpublished) data have reported for another apoptosis
system that this low percentage DAPI-abnormal cell is not
due to a specific apoptosis-inducing agent, but due to
20   transfection events. Also, the experiments shown below
prove this statement. In similar experiments, 60-90% of
malignant cells containing apoptin were apoptotic [Zhuang
et al., 1995, 1995a,b). The localization of apoptin in
these primary cells also differed from the localization in
25   tumor cells. In all of the normal cells, apoptin was found
in the cytoplasm, and not in the nucleus as has been
observed for various tumor cell lines (Zhuang et al.,
1995, 1995a,b). These results suggest that apoptin fails
to induce apoptosis in various cultured human non-
30   transformed, non-tumorigenic cells, and that the cellular
location of apoptin is important for its apoptotic
activity.

<u>Expression of apoptin in human normal cells and their</u>
<u>malignant derivatives.</u>

We also examined the response to apoptin expression
in tumor cell lines versus that in the normal cells from
5   which they had been generated. To that end, diploid skin
fibroblasts and keratinocytes from normal individuals and
their tumorigenic counterparts were transfected
transiently with pCMV-fs, expressing full-sized apoptin,
pCMV-tr, expressing truncated apoptin or a plasmid
10   encoding desmin (pCMV-des). Desmin has no apoptotic
activity (Menke et al. unpublished data), and is used as a
negative control. Five days after transfection, the
percentage of apoptin-positive VH10 fibroblasts (Figure 5)
and FSK-1 keratinocytes (Figure 6), which had become
15   apoptotic was not above 15%. This level of aberrantly
DAPI-stained cells was similar for cells containing
truncated apoptin or desmin. The low-level of apoptosis in
these apoptin-positive cells may be due not to apoptin-
specific induction of cell death, but to transfection
20   events.

To examine whether tumorigenic fibroblasts and
keratinocytes were susceptible to apoptin, NW18 and SCC-15
were transfected with plasmids encoding apoptin (full-
sized or truncated) or desmin. Apoptin and, to a lesser
25   extent, truncated apoptin could induce apoptosis in the
NW-18 (Figure 5) and SCC-15 (Figure 6) tumor cells. Up to
65-75% of these apoptin-positive cells was apoptotic at
five days after transfection, which is in the similar
range as reported for apoptin-induced cell death in
30   osteosarcoma cell lines (Zhuang et al., 1995a). The level
of abnormal DAPI-stained desmin-positive NW-18 and SCC-15
cells was not significantly higher than that among normal
fibroblasts and keratinocytes (Figures 5 and 6).

The differential activity of apoptin in normal and
35   tumor cells cannot be explained by different proliferation
rates, as these are similar for VH10 fibroblasts and Saos-
2 osteosarcoma cells.

Our observations show that apoptin does not induce
apoptosis in normal fibroblasts and keratinocytes, but
does in their tumorigenic derivatives.

5   Expression of apoptin in human transformed cells.

Is tumorigenicity required for apoptin to induce cell
death, or is mere transformation of a cell sufficient? To
answer this question we have examined the effect of
apoptin in transformed,non-tumorigenic fibroblasts and
10   keratinocytes. Apoptin, truncated apoptin, and desminwere
transiently expressed in SV40-transformed fibroblasts,
prior (Pre) or post (Post) immortalization, in SV40-
transformed and immortalized SVK14 keratinocytes, and in
spontaneously transformed HaCaT keratinocytes. Apoptin was
15   able to induce apoptosis in all these types of
transformed cell, to an extent similar to that in tumor
cells (Figures 5 and 6). The percentage of cells
containing (truncated) apoptin which had become apoptotic
was significantly higher than in desmin-positive cells.
20   These data imply that apoptin can induce apoptosis in both
malignant and transformed cells.

Some chemotherapeutic agents and radiation can induce
apoptosis in transformed cells but fail to do so in
untransformed cells (Thompson, 1995, McDonell et al.,
25   1995). Transformation seems to cause changes that make a
cell more sensitive to apoptotic stimuli. Some other
proteins are known to kill tumor cells specifically. The
parvovirus structural protein NS-1 induces cell lysis
specifically in neoplastic cells (VanAcker and Rommelaere,
30   1995). Also, a bcl-xs-expressing adenovirus has been
constructed that induces apoptosis in human breast- and
colon-carcinoma and neuroblastoma cells, but not in human
normal hematopoietic cells or in the human leukemia cell
line K562 (Clarke et al., 1995).

<u>Stable transfection of VH10 cell with a plasmid ecoding
apoptin.</u>

　　　　To exclude that apoptin has a minor but significant
apoptotic activity in normal VH10 cells, these cells were
5　stably transfected with pCMV-fs or with pCMV-neo-Bam as a
control. We obtained a similar amount of colonies in both
transfections. The resulting cells, stably transfected
with pCMV-fs, expressed apoptin which was located in the
perinuclear region (data not shown). Therefore, it can be
10　concluded that apoptin cannot induce apoptosis or inhibit
the growth of normal VH10 cells in any other sense.

<u>Cellular localization of apoptin in normal versus
transformed and malignant cells.</u>

15　　　　Apart from the different apoptotic activities of
apoptin in malignant and transformed cells versus normal
cells, we have also observed differences in the cellular
localization of apoptin in these cell types. In
transformed and malignant cells, before the apoptotic
20　morphological changes are oticeable, apoptin is,
distributed as fine granules, mainly in the nucleus. After
the cells had undergone apoptosis, apoptin was aggregated
in the nucleus. In contrast, the location of apoptin in
normal fibroblasts and keratinocytes is mainly in the
25　cytoplasm, concentrated round the nucleus, both as small
granules and larger aggregates. In primary HUVECs, HSMCs
and T cells, apoptin was also localized in these
characteristic perinuclear structures. Thus far, in all
malignant or transformed cell lines studied, apoptin had a
30　nuclear location, whereas in all normal cells analysed
thusfar, it had not.
　　　　Others have proposed that transformed cells have
undergone loss-of-function mutations, as a result of which
a normally functioning inhibitor has been switched off.
35　Due to these changes, nuclear transport of proteins may be
promoted or hindered in cancer cells (Csermely et al.,
1995). It is possible that in normal cells apoptin is

associated to and/or modified by one or more cellular
factor(s), resulting in its location within perinuclear
structures. The loss of such (functional) factor(s) in
malignant cells might allow apoptin to enter the nucleus.

5   Apoptin harbors not only putative nuclear-import
sequences (Noteborn et al., 1991, 1994, 1995, Zhuang et
al., 1995, 1995a,b, but also an amino-acid region
resembling nuclear export signals (position 33-46:
IRIGIAGITITLSL), similar to that of the protein kinase

10   inhibitor (PKI) and the HIV-Rev protein (Wen et al.,
1995, Fischer, et al., 1995, Gerace, 1995). It might well
be that this potential nuclear export signal cannot be
recognized in the various analysed malignant and
transformed cell lines.

15   The results described here indicate that the nuclear
localization of apoptin is important for its ability to
induce apoptosis. This is in agreement with the
observation that truncated apoptin, which has a reduced
apoptotic activity (Figures 5 and 6), is partially in the

20   cytoplasm. Electron microscopic studies with chicken
mononuclear cells revealed that apoptin co-localizes with
the cellular chromatin (Noteborn et al., 1994). The
interaction of apoptin with chromatin could result in
unwinding of its superstructure. The latter phenomenon has

25   been described for rat ventral prostate cells, which
became apoptotic after castration of the rats (Kyprianou
and Isaacs, 1989).

Apoptin as an anti-tumor agent.

30   Our results indicate that apoptin is an anti-tumor
agent. Firstly, apoptin is specifically active in
malignant and transformed cells, but, at least in vitro,
not in the normal cells tested. Therefore, the toxic
effect of apoptin treatment might be very low. Secondly,

35   apoptin induces apoptosis in a p53-independent manner. The
fact that several chemotherapeutic agents lose this
capacity to induce apoptosis in a large number of tumors

seems to be related to a disruption of p53 function.
Therefore, induction of a p53-independent apoptotic
pathway might be a useful approach as an alternative
candidate for tumor therapy. Furthermore, apoptin
5   apparently is not blocked by Bcl-2, which is known to be
involved in the development of, e.g., leukemic tumors and
which can inhibit apoptosis induced by chemotherapeutic
agents. In addition, we have observed that BAG-1, a Bcl-
2-related protein, is not able to block apoptin-induced
10   apoptosis.

Description of the figures

Figure 1 shows the effect of expression of E1B-21kD
and Bcl-2 on the induction of apoptosis by VP3 in p53-
15   minus Hep3B cells. The percentage given is that of the
apoptin-positive cells which are apoptotic 6 days after
transfection. The cells were co-transfected with 2.5 ug of
pCMV-VP3 and 5 ug of pCMV-neo-Bam (open bars), 5 ug of
pCMV-E1B21 (hatched bars), or 5 ug of pCMV-Bcl2 plasmid
20   DNA (dotted bars). At least 3 independent experiments were
carried out. Per experiment at least 200 apoptin-positive
cells were examined.

Figure 2 shows the effect of E1B-21K and Bcl-2
proteins on the induction of apoptosis by p53 in the Hep3B
25   cell line. The cells were co-transfected with 2.5 Êug of
pCMV-p53 and 5 Êug of pCMV-neo-Bam, 5 ug of pCMV-E1B21K,
or 5 ug of pCMV-Bcl2 plasmid DNA. Two independent
transfections were carried out. The cells were analysed 4
or 5 days after transfection. The percentage given is that
30   of p53-positive cells which are apoptotic. Per experiment
at least 200 p53-positive cells were examined.

Figure 3 shows the effect of Bcl-2 and Bag-1
expression on the p53-induced apoptosis or apoptin-
induced apoptosis. Saos-2 cells were co-transfected with
35   2.5 ug of pCMV-fs, and 5 ug of pCMV-Bcl-2, pCMV-Bag-1, or
PCMV-Bag-1 and pCMV-Bcl-2. In a parallel experiment, the
cells were co-transfected with 2.5 ug of pCMV-p53 and 5 ug

pCMV-Bcl-2, pCMV-Bag-1 or pCMV-Bag-1 and pCMV-Bcl-2. As
controls, pCMV-p53 or pCMV-fs were co-transfected with
pCMV-neo-Bam. At least, 3 independent experiments of both
series were carried out. The cells were harvested 4 days
5   after transfection. The percentage is given of the p53- or
apoptin-positive cells which have become apoptotic. Per
experiment at least 200 cells have been examined.

       Figure 4 shows the effect of the ICE-inhibitor crmA
on the induction of p53- or apoptin-induced apoptosis.
10   Saos-2 cells were co-transfected with 2.5 ug pCMV-fs and 5
ug pCMV-crmA (+crmA) or pCMV-neo-Bam (-crmA). In parallel,
cells were co-transfected with 2.5 ug pCMV-p53 and 5 ug
pCMV-crmA or pCMV-neo-Bam. Two independent experiments
were carried out. The cells were harvested 5 days after
15   transfection. The percentage is given of the p53- or
apoptin-positive cell which have become apoptotic. Per
experiment, at least 200 positive cells were examined.

       Figure 5 shows the apoptin activity in normal versus
transformed or malignant human fibroblasts. The percentage
20   of cells that stained abnormally with DAPI is given as a
relative measure for apoptosis in normal VH10 versus
transformed (Pre, Post) and tumor (NW18) fibroblasts,
transiently transfected with pCMV-fs, pCMV-tr or pCMV-des.
Cells were fixed five days after transfection and analysed
25   by indirect immunofluorescence. Results are the means of
at least three independent experiments. In each
experiment, at least 200-full-sized or truncated apoptin-
or desmin-positive cells were examined. Cells were fixed 5
days after transfection and analysed by indirect
30   immunofluorescence.

       Figure 6 shows the apoptin activity in normal versus
transformed or malignant keratinocytes. The percentage of
cells that stained abnormally with DAPI is given as a
relative measure for apoptosis in normal keratinocytes
35   (FSK-1) versus transformed (SVK14, HaCAT) and tumor (SCC-
15) ones, transiently transfected with pCMV-fs, pCMV-tr,
or pCMV-des. Cells were fixed five days after transfection

and analysed by indirect immunofluorescence. Results are the means of at least three independent experiments. In each experiment, at least 200-full-sized or truncated apoptin⁻ or desmin-positive cells were examined.

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CLAIMS

1.   A method for distinguishing between transformed
and/or malignant and/or tumor cells and normal cells
comprising the steps of providing said cells with the
viral protein 3 (VP3; apoptin) and detecting the
5   localization of said viral protein in said cells.
2.   A vehicle for delivering a nucleic acid of interest
to a target cell, said vehicle further comprising a gene
encoding apoptin or a functional equivalent thereof.
3.   A conjugate for targeted tumor therapy comprising a
10   targeting moiety having binding affinity for a molecule
associated mainly, but not exclusively with the surface of
a tumor cell and apoptin or a functional equivalent
thereof.
4.   A vehicle delivering a tumoricidal substance or a
15   gene encoding a tumoricidal substance to a molecule
associated mainly but not exclusively with a tumor cell,
characterized in that the tumoricidal substance is apoptin
or a functional equivalent thereof.
5. Apoptin or a functional equivalent thereof for use in a
20   method of eliminating cells of a target cell population
whereby the method is mainly, but not entirely specific
for the cells of the target population, whereby apoptin or
its functional equivalent is the ctotoxic agent.
6.   Apoptin or a functional equivalent thereof for use in
25   a method of eliminating cells of a target cell population
whereby the cells of said population are not sensitive to
other apoptosis inducing agents.
7.   Apoptin or a functional equivalent thereof provided
with a nuclear localization signal.
30   8. Apoptin or a functional equivalent according to claim 8
for use in a method for elimination of cells of a
population of target cells.
9.   A nucleic acid encoding an apoptin or a functional
equivalent thereof according to claim 8.
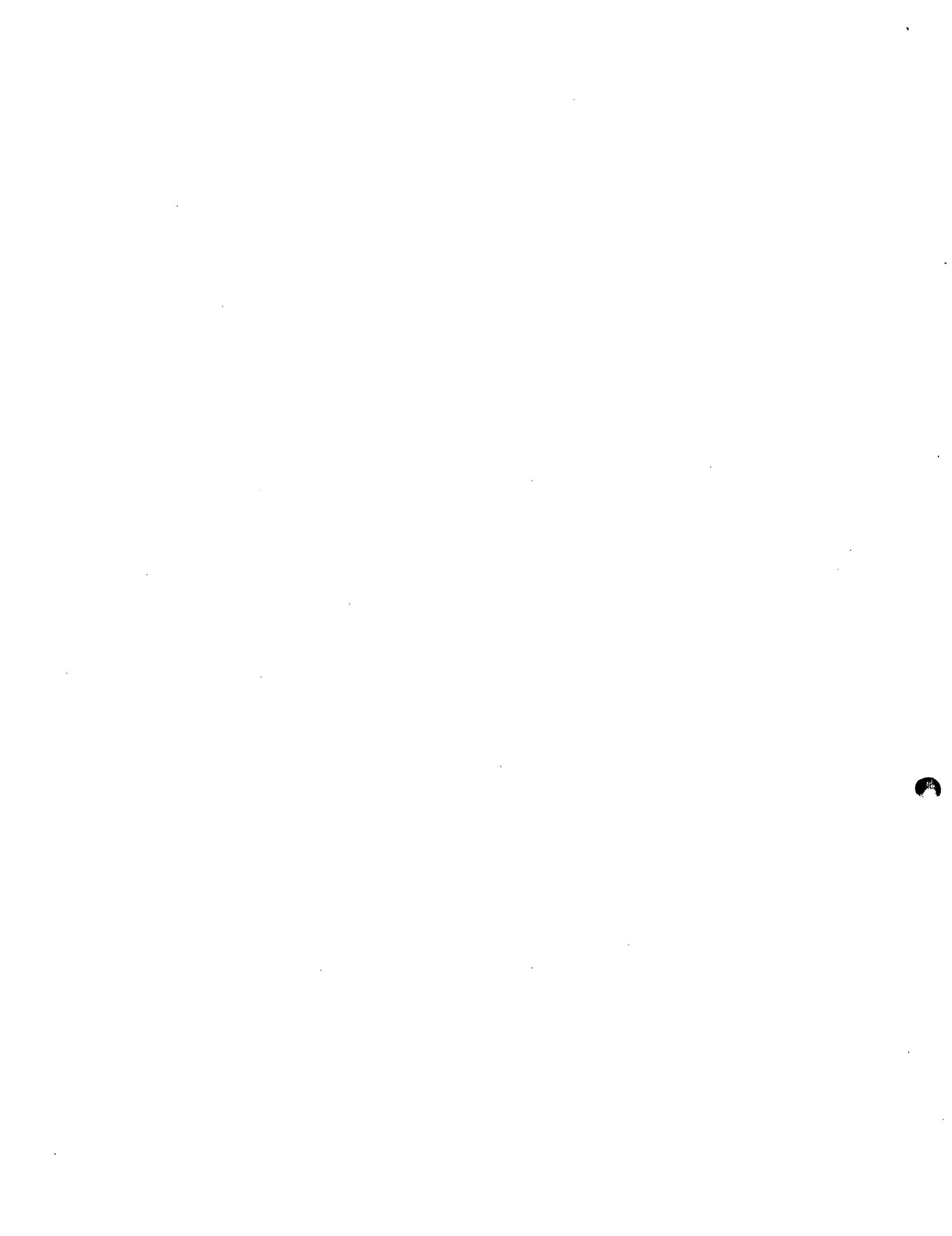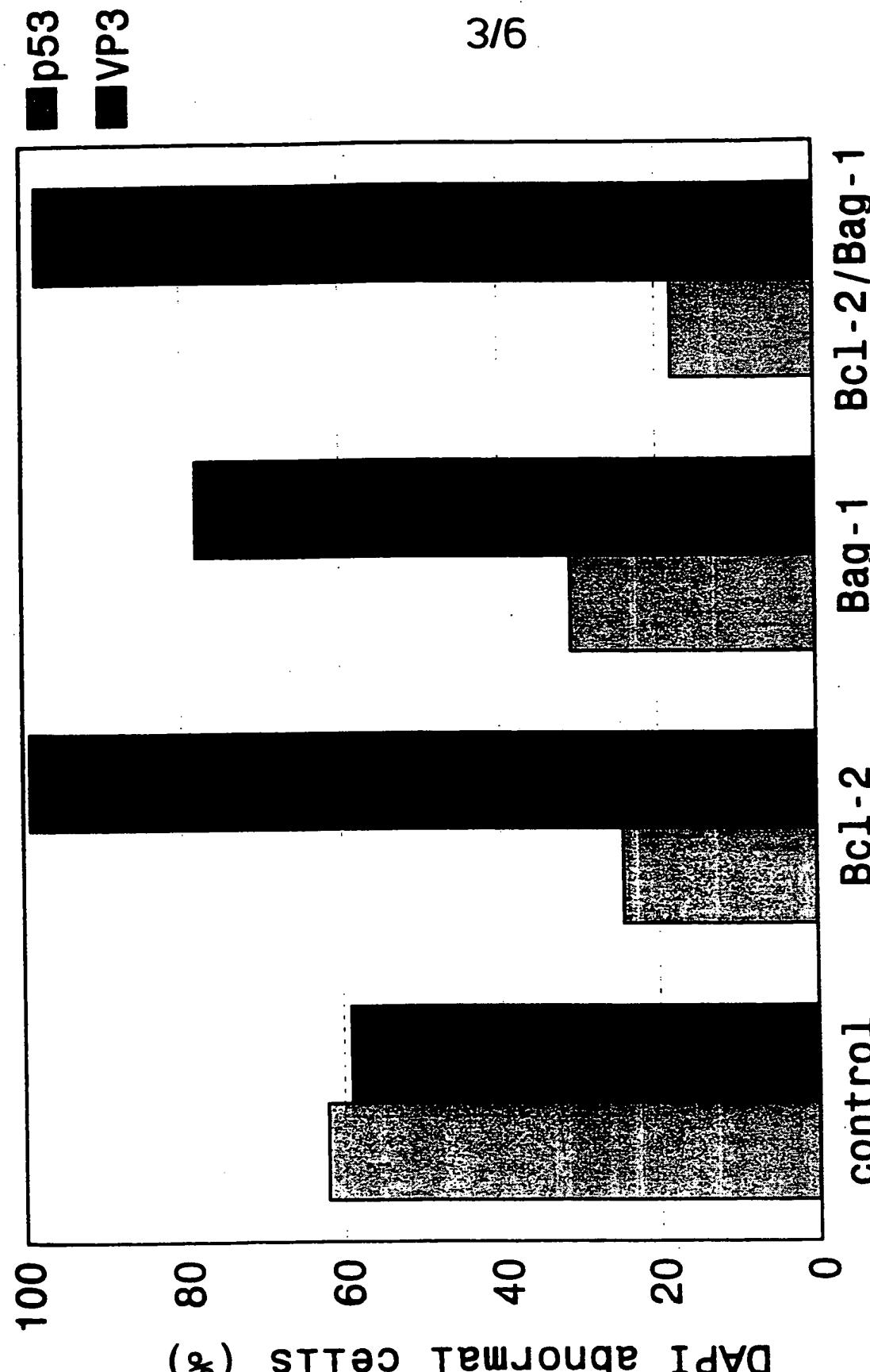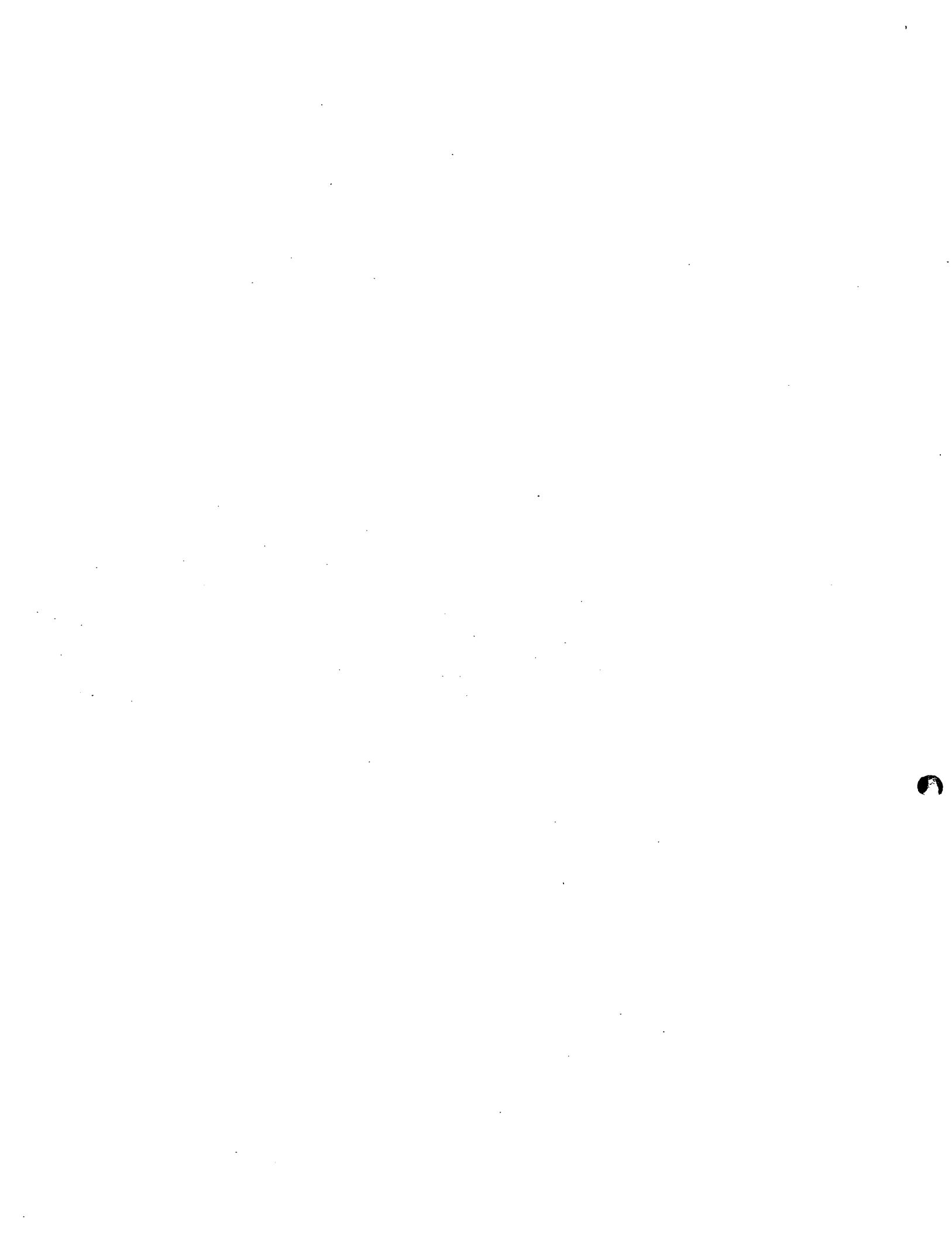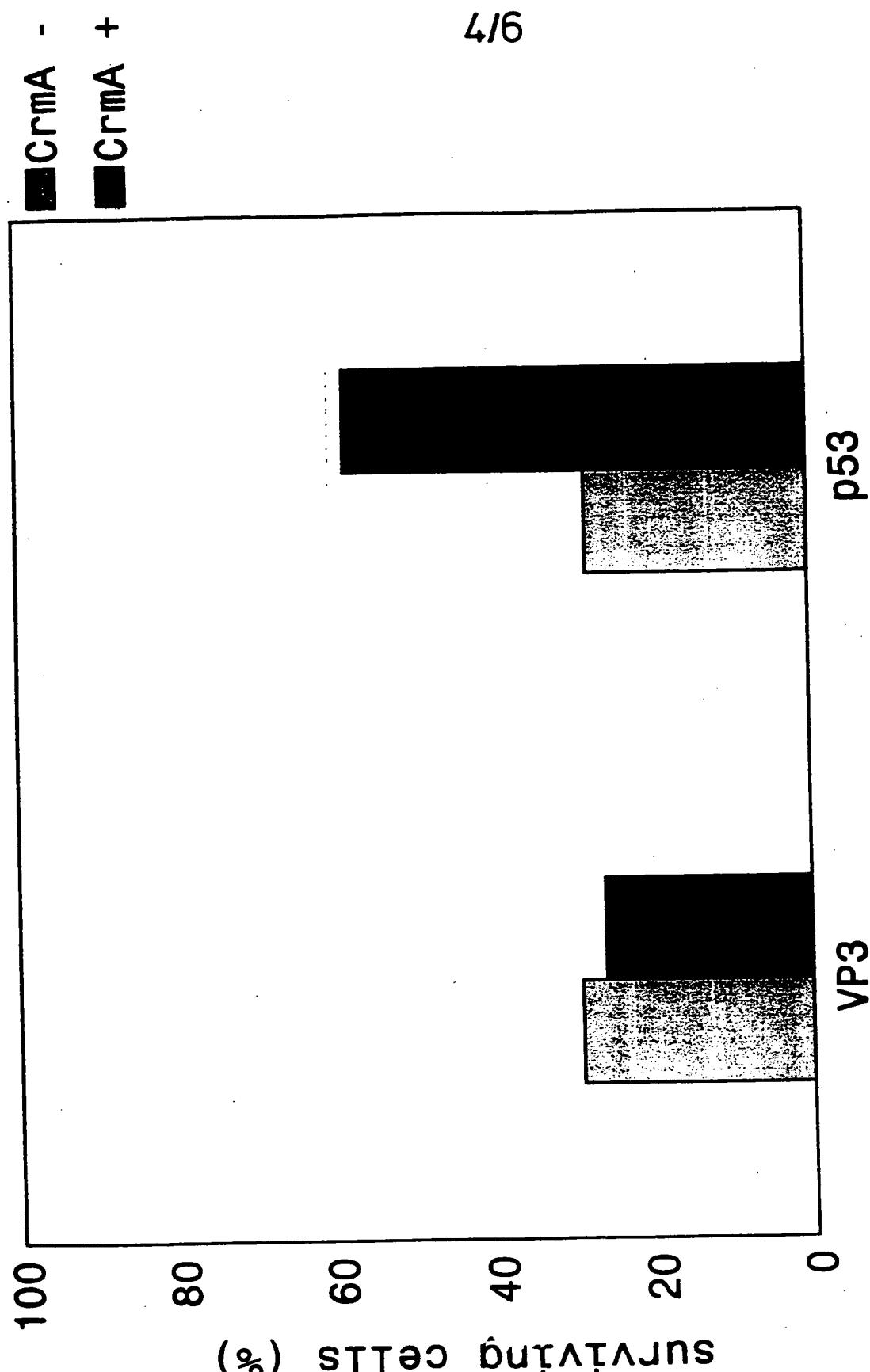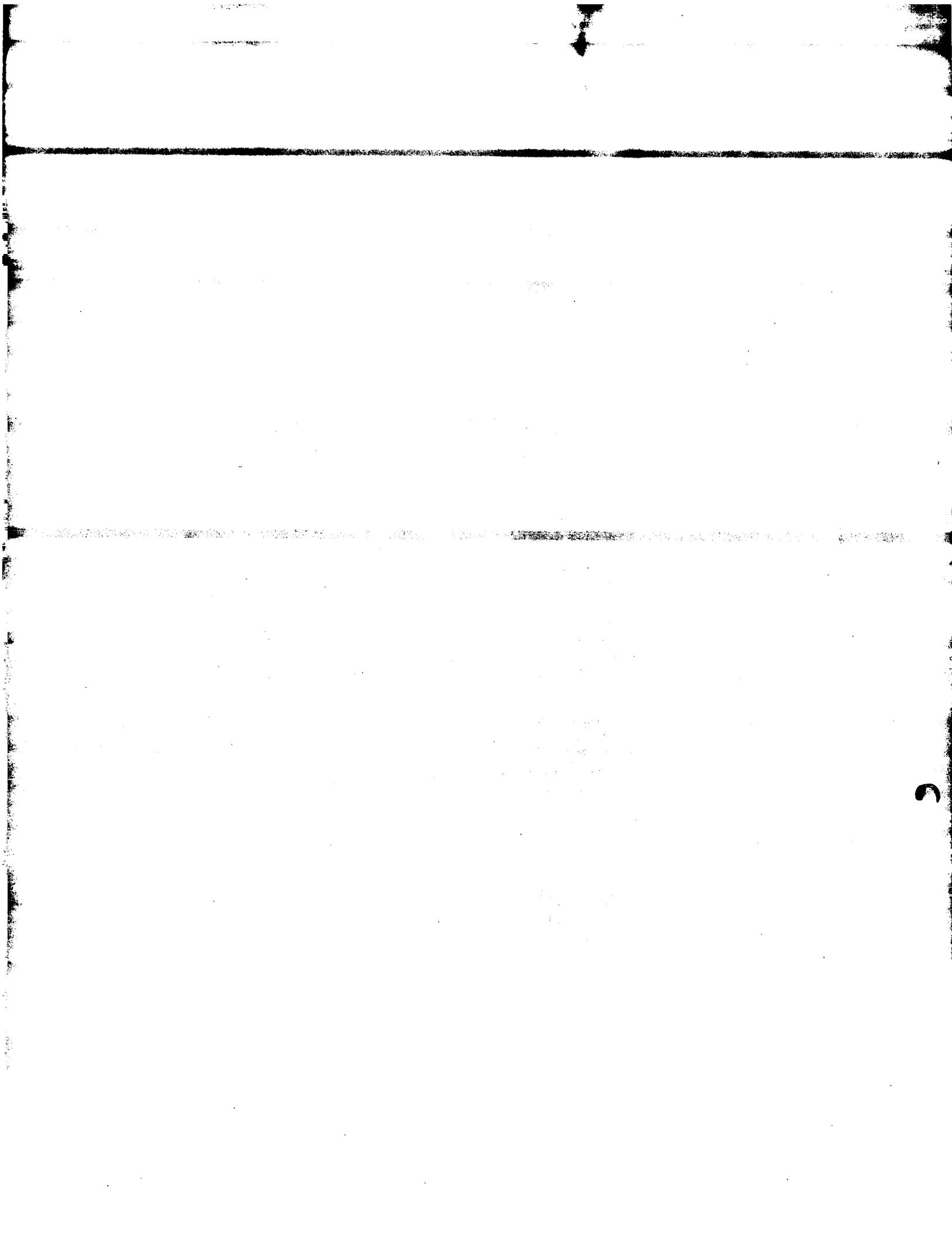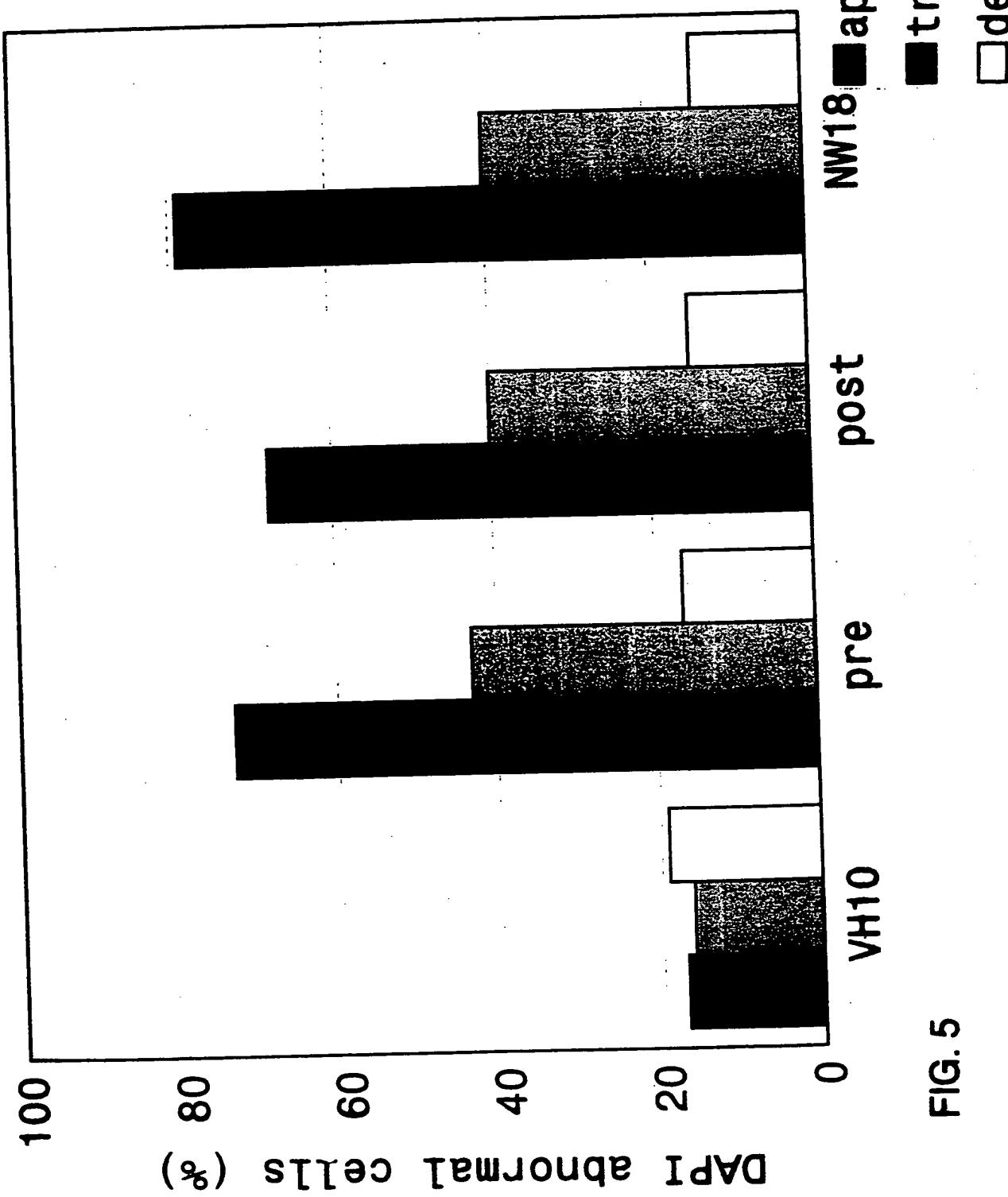
**SUBSTITUTE SHEET (RULE 26)**

FIG. 1

FIG. 2

FIG. 3

FIG. 4

5/6

FIG. 5
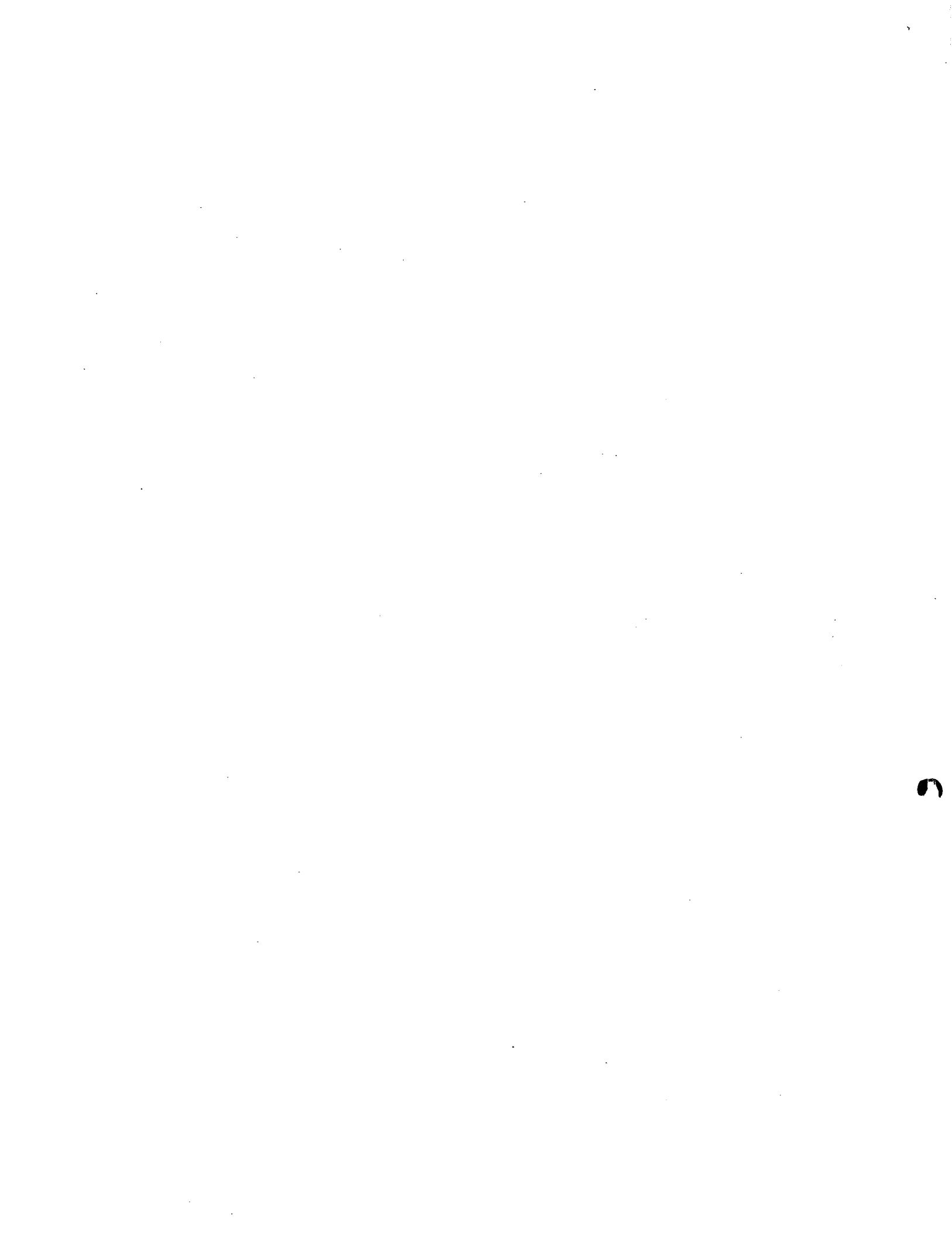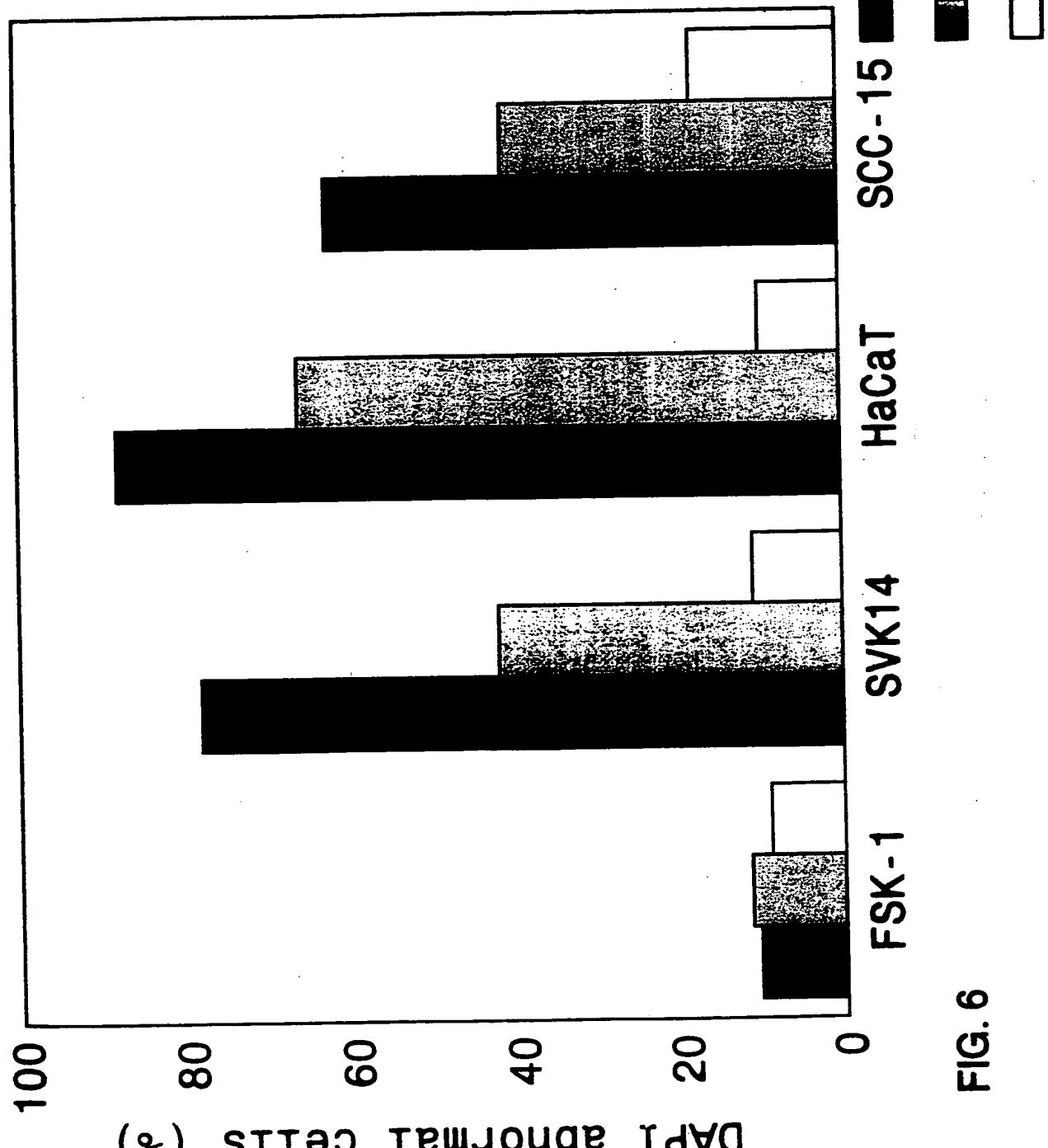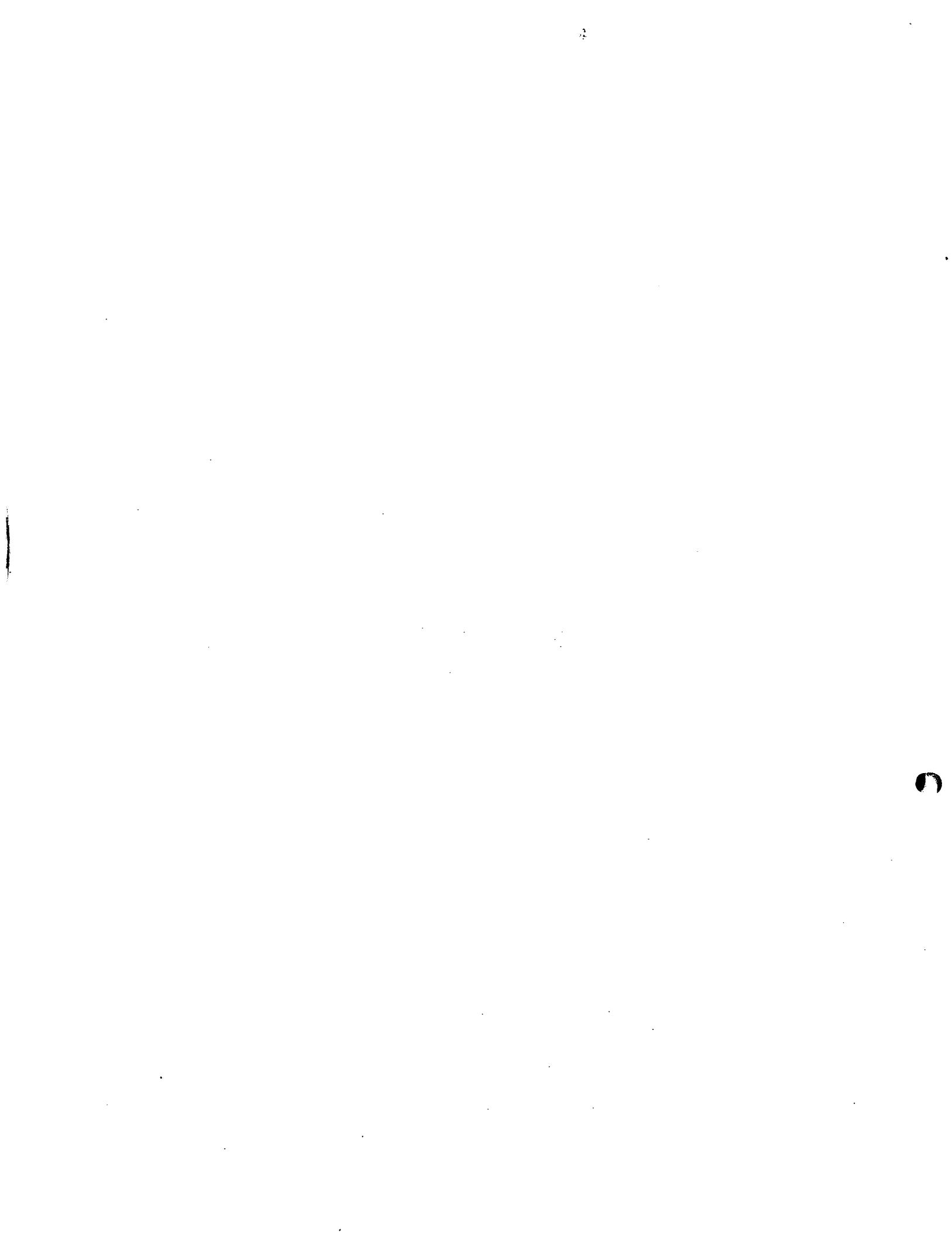
FIG. 6

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   G01N33/574   A61K38/16   C12N15/34   C07K14/01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C07K   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,95 03414 (AESCULAAP BV ;NOTEBORN MATHEUS HUBERTUS MARI (NL); KOCH GUUS (NL)) 2 February 1995 see page 1, line 31 - line 34 see page 8, line 14 - page 9, line 36 see page 26, line 33 - page 27, line 2 see page 28, line 1 - page 30, line 22 ---	2-9
X	CANCER RESEARCH, vol. 55, February 1995, pages 486-489, XP000602162 ZHUANG S.-M. ET AL: "Apoptin, a protein derived from chicken anemia virus, induces p53-independent apoptosis in human osteosarcoma cells" cited in the application see the whole document, especially page 486, abstract and page 488 right column ---	2-9

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 September 1996	2 7. 09. 96

| Name and mailing address of the ISA
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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF VIROLOGY, vol. 68, no. 1, 1994, pages 346-351, XP002013505 NOTEBORN M.H.M. ET AL: "A single chicken anemia virus protein induces apoptosis" see the whole document	9
X	JOURNAL OF VIROLOGY, vol. 65, no. 6, 1991, pages 3131-3139, XP002013506 NOTEBORN M.H.M. ET AL: "Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle" see the whole document	9
X	AVIAN PATHOLOGY, vol. 24, 1995, pages 11-31, XP000577477 NOTEBORN M.H.M. ET AL: "Chicken anemia virus infection: molecular basis of pathogenicity" cited in the application see the whole dicument, especially pages 22-24 "A single CAV protein induces apoptosis"	2-9
X,P	LEUKEMIA, vol. 9, no. 1, October 1995, pages s118-s120, XP000602147 ZHUANG S.-M. ET AL: "Apoptin, a protein encoded by chicken anemia virus, induces cell death in various human hematologic malignant cells in vitro" cited in the application see the whole document	1-9

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INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   Please see Further Information sheet enclosed.

2. [ ] Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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FURTHER INFORMATION CONTINUED FROM   PCT/ISA/210

Remark : Although claim 1 (partially , in so far as the method is carried out
in vivo) is directed to a diagnostic method practised on the
human/animal body the search has been carried out and based on
the alleged effects of the compound.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9503414	02-02-95	NL-A-	9301272	16-02-95
		AU-A-	7547394	20-02-95
		CA-A-	2167578	02-02-95
		ZA-A-	9405275	24-02-95